

Nanogram Quantification of Nonpolar Lipid Classes in Environmental Samples by High Performance Thin Layer Chromatography

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A sensitive, simple, and rapid high performance thin layer chromatography (HPTLC) method that quantifies nanogram amounts of complex nonpolar lipid classes in environmental samples with a minimum of sample preparation is presented. The derivatization method is lipid-specific and insensitive to carbon number or the degree of unsaturation of the fatty acids composing the lipid class compounds. Nonpolar lipid classes ranging from 10 to over 500 ng easily may be quantified in the same sample run. The coefficient of variation for sample replicates on different plates ranged from 2.3% to 5.9%. Accuracy of the method is better than 15%. The nonpolar lipid class composition of <53 μm oceanic particles in a vertical depth profile of the upper 600 meters of the Slope Water was determined to illustrate the HPTLC method. The observed changes in particulate lipid class composition indicate that secondary production by deep-living organisms significantly alters suspended particle composition with depth in the water column.

Lipids 23, 493-500 (1988).

Lipid classes are useful tracers of the sources and transformations of organic carbon in particulate material in the water column (1-4), sediments (5-7) and other environmental materials. However, complete characterization of the lipid classes in these samples has been limited by the lack of simple methods to resolve complex mixtures of plant, animal and microbial lipids, the time required for analyses by existing methodologies, and the small amount of material often available. In our studies of oceanic particulate material, we required an analytical technique that could adequately resolve and quantify sub- μg amounts of complex lipid classes yet was simple enough for routine analyses. No existing method satisfied these requirements.

Ideally, a method used to resolve and quantify complex lipid class composition in such small environmental samples should be very sensitive yet require a minimum amount of sample manipulation. Previous studies have used either column (1-4) or thin layer chromatography (5,7-9) to separate lipid classes, followed by gravimetric, spectrophotometric or gas chromatographic analyses to quantify the lipid fractions. These methods are tedious and time-consuming and allow only a few samples to be processed per day. The multiple steps involved can result in sample loss, fractionation or degradation. Moreover, complex lipid classes often overlap in the fractions so additional steps are required to purify each lipid class. Tens to hundreds of μg of total lipids may be needed to

accurately quantify minor lipid classes in a complex sample by these methods. High performance liquid chromatography (HPLC) has been used to separate lipid classes (10,11), but quantitative measurement of the nonpolar lipid classes has not been achieved. Alternatively, lipid classes have been separated on thin-layer plates and derivatized *in situ* to form compounds that can be quantified by scanning densitometry (6,8,12-14). This method is more expedient than the above methods but requires μg amounts of lipid classes and has limited precision due to incomplete separation of complex lipid classes and high background noise. More recently, the Iatroscan/Chromarod system has been used to determine the lipid class composition in a variety of materials (15-21). While the Iatroscan/Chromarod has found widespread use in routine lipid analysis, the system has limitations in sub- μg analyses of complex lipids due to problems with nonreproducibility, poor precision, lack of sensitivity and nonlinearity of response (21-23). None of these techniques were sufficiently sensitive and expedient for routine analyses of lipid class composition in our samples.

However, recent advances in quantitative high performance thin layer chromatography (HPTLC) suggested that this method might be ideally suited for sub- μg quantification of complex lipid classes. Quantitative HPTLC combines the many inherent advantages of TLC with high resolution and subnanogram detection limits for a wide variety of compounds (24,25). The newly developed preadsorbent sample application zone on HPTLC plates eliminates the sample application difficulties previously encountered in HPTLC and serves to cleanup and narrowly focus the sample at the preadsorbent zone/silica gel interface, thus enhancing the separation of complex lipids (26). The development of fluorescent-inducing reagents (27-33) has enabled nanogram quantities of the major lipid classes to be detected easily in samples of uniform biological origin such as blood serum, amniotic fluid and tissue extracts (33-36). This study investigated whether HPTLC methods also were suitable for quantitative analyses of complex lipid classes of unknown composition in environmental samples.

In this paper, we show that HPTLC is a rapid, sensitive, quantitative and reproducible technique for sub- μg analyses of complex lipid classes in oceanic particulate material. The HPTLC method presented here generally is applicable to most materials. The resolution of complex lipid classes is more complete, and the optimal concentrations for quantitative work are nearly two orders of magnitude less with HPTLC than with currently employed methods, including the Iatroscan/Chromarod system. In addition, the method is very rapid and allows up to 50 analyses of crude lipid extracts to be easily completed per day.

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Materials and reagents. HPTLC silica gel plates (10 \times 10 cm) with preadsorbent sample application zones were

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Abbreviations: MG, monoacylglycerol; DGE, diacylglycerol ether; DG, diacylglycerol; CF, cholesteryl formate; FAL, fatty alcohol; FAME, fatty acid methyl ester; FFA, free fatty acid; POC, particulate organic carbon; TG, triacylglycerol; ST, sterol; STE, steryl ester; WE, wax ester; FID, flame ionization detector; HPLC, high performance liquid chromatography; HPTLC, high performance thin layer chromatography; TLC, thin layer chromatography.

obtained from Merck (Darmstadt, FRG). Lipid class standards were obtained from Nu-Chek Prep, Inc. (Elysian, MN). Free fatty acids were obtained from Sigma Chemical Co. (St. Louis, MO). Aminopropyl Bond-Elut disposable chromatography columns with stainless steel frits were used for preparative separation of lipid classes and were obtained from Analytichem International (Harbor City, CA). Prechanneled thin layer chromatography (TLC) plates with preadsorbent zones were used for purification of the lipid class fractions and were obtained from Whatman (Clifton, NJ). All solvents were chromatography grade.

Sample collection and preparation. Oceanic particulate material was collected from the upper 1000 meters using large-volume in situ filtration (37). Samples described in this paper are from the Slope Water 200 km off the coast of Virginia and were collected in June 1982. Further details of the collection method and the sample site can be found in Bishop et al. (38).

The filter samples were subsampled within two hr of retrieval. Each subsample represented about 10% of the total material collected by filtration and contained approximately 2 to 5 mg of organic carbon. Subsamples were placed in 20-ml test tubes and filled to the top with chloroform/methanol (2:1, v/v). The antioxidant BHT (0.05%, w/v) was added to the sample tubes approximately six months later. Samples were stored at -20°C until analyzed. There was no evidence of compound oxidation or hydrolysis, or fatty acid methyl ester formation in the samples during prolonged storage.

Lipids were extracted using a modification of the procedure described by Folch et al. (39). Cholesteryl formate was added as an internal TLC standard before extraction to correct for sample losses and spotting variability. The chloroform/methanol solvent in the sample tubes was vacuum-filtered through a fritted funnel into a separatory funnel, and the sample tube was refilled with fresh chloroform/methanol (2:1, v/v). The sample tube then was ultrasonicated in a cup horn at 12°C at 100 watts for 45 sec using a Sonifier cell disruptor (Heat Systems-Ultrasonics, Plainview, NY). This solvent then was filtered through the fritted funnel into the separatory funnel and the filters rinsed with solvent. Aqueous KCl (0.88%, w/v) (1/4 the volume of the total solvent) then was added to the separatory funnel and the lipids extracted into the lower organic phase. The lower phase then was rotary evaporated at 30°C to the point of dryness, redissolved in chloroform and passed through a mini-cleanup column containing sodium sulfate to remove any residual water. The final volume of the crude lipid extract then was adjusted to 2 ml with chloroform.

Standard lipid classes. Simple lipid class mixtures comprised of monoolein (MG), diolein (DG), triolein (TG), oleic acid (FFA), methyl oleate (FAME), stearyl oleate (WE), cholesterol (ST) and cholesteryl oleate (STE) were prepared in concentrations ranging from 1 to 160 ng/ μl . The internal standard cholesteryl formate also was added to these standard mixtures to correct for spotting variability and evaporation. The amount of cholesteryl formate added to each standard was adjusted to produce an average final concentration of 50 ng per 10 μl spot.

Complex lipid classes purified from a larger sample of oceanic particulate material were used to calibrate the response of simple lipid class standards with the complex

lipid classes found in samples. The lipid classes in the oceanic particulate material were separated using disposable aminopropyl Bond-Elut chromatography columns (40). The lipid class fractions then were purified by TLC using hexane/ether/acetic acid (80:20:1, v/v/v) as the developing solvent.

These sample lipid classes were independently quantified by capillary gas chromatography. The free fatty acid and triacylglycerol fractions were transesterified with 5% methanolic HCl (41) at 50°C for 12 hr to form fatty acid methyl esters. Nonadecanoic acid was added as an internal standard before transesterification. The fatty acid methyl esters were analyzed using a Carlo Erba 5160 gas chromatograph equipped with on-column injection. Chromatography was performed on a 50 m \times 0.25 mm CP Sil 88 column (Chrompack, Bridgewater, NJ) programmed from 110 to 220°C at $5^{\circ}\text{C}/\text{min}$. The GC was interfaced to an Apple IIe computer using a SOFT 510 system (Data Acquisition Systems, Boston, MA) and peaks were quantified using DYSC chromatography software (Dynamic Solutions Corp., Pasadena, CA). The TG concentration was corrected to include the additional weight of the glycerol moiety.

Thin layer chromatography. HPTLC plates were predeveloped to remove impurities and binder material that impart a high background fluorescence. Plates first were predeveloped in methanol and then in the same polar solvent later used for development (methyl acetate/n-propanol/chloroform/methanol/43 mmol/l aqueous potassium chloride, 25:25:25:10:9, v/v/v/v/v) (35). A thin line was then scraped across the top of the plate about 3 mm from the top to prevent these impurities from back-diffusing into the upper part of the plate during later developments. The plates were then dried at room temperature and activated in a 110°C oven for one hr before chromatography. Plates were handled carefully to maintain a dust-free, unmarred surface.

Seven samples and two standard mixtures were applied to each 10 \times 10 cm plate. The lipid class concentrations of the two standard mixtures bracketed the expected lipid class concentrations in the samples. Either 5 or 10 μl of lipid extract or standards were applied quantitatively to the preadsorbent zone about 5 mm from the end of the plate using an Oxford 5 μl fixed volume micropipette with a Teflon tip (Sherwood Medical, St. Louis, MO). This volume can be rapidly yet quantitatively applied to the preadsorbent zone of HPTLC plates and eliminated the need for a sample concentration step. Each sample was applied as a line of overlapping spots perpendicular to the preadsorbent interface and parallel to the direction of development. Spot diameter was maintained at <4 mm. This application method enables the sample lipids to be focused in a narrow band at the preadsorbent interface at the beginning of development. A gentle stream of nitrogen was used to evaporate the excess solvent during application. Although this application method results in slightly higher sample concentrations at the edges of the sample band than in the center, the uneven distribution has negligible effect on quantitation provided the width of the sample band is less than the slit height used for densitometry so that the entire sample band is scanned.

Plates were developed in saturated TLC tanks by one of two methods. Method I: the plate was developed in

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hexane/heptane/ether/acetic acid (63:18.5:1, v/v/v/v) (35) for 10 min (6 cm), dried with a stream of unheated air using a hairdryer for two min, then developed twice in hexane/benzene (3:1, v/v) for 15 min.

While this developing method gave good resolution, the silica gel surface near the preadsorbent zone interface frequently was uneven, which caused problems with quantification by scanning densitometry. In addition, development by Method I did not completely separate the internal standard from the FAME region and wax esters from the steryl ester region. Therefore, later experiments used a different developing scheme (Method II) that moved the nonpolar lipids farther away from the preadsorbent zone interface and increased the resolution between the lipid classes. We also found Method II gave slightly lower backgrounds.

Method II: this method used a series of three developing solvents. The plate was first developed in carbon tetrachloride for eight min (5 cm) to narrowly band the sample lipids at the preadsorbent/silica gel interface and to remove the antioxidant BHT, which migrates with the solvent front, away from the sample zone. Next the plate was developed in methyl acetate/1-propanol/chloroform/methanol/43 mmol/l KCl (25:25:25:10:9, v/v/v/v/v) for two min (0.4 cm) to separate the neutral lipids from the polar lipids and to remove the neutral lipids from the interface zone. The neutral lipids then were separated using double development in hexane/heptane/ether/acetic acid (63:18.5:18.5:1, v/v/v/v/v) (35) for eight min (6.5 cm). This double development increased the resolution of the lipid classes. Finally, the plate was developed in carbon tetrachloride for 30 min to more completely separate the internal standard cholesterol formate from fatty acid methyl esters, and steryl esters from wax esters. The plate was dried under a stream of unheated air using a hairdryer for two min between developments.

Derivatization. The lipid classes were derivatized using the method of Schmitz et al. (35). The developed plate was thoroughly air-dried and then dipped in the manganese chloride-sulfuric acid derivatizing reagent (3.2 g MnCl_2 , 32 ml conc. H_2SO_4 , 480 ml H_2O , 480 ml methanol) (27)

for 20 sec. The back of the plate then was blotted to remove excess reagent and the plate dried with a cold hairdryer for five min to evaporate the solvents. The plate was placed in a 110 C oven for exactly 40 min to form fluorescent derivatives of the lipid classes. Following derivatization, plates were stored in a dessicator until scanned.

Densitometry. Plates were scanned using a Camag II scanning densitometer (Camag, Inc., Wrightsville Beach, NC) in the reflectance mode. The monochromator on the mercury light source was set for an excitation wavelength of 366 nm and 10 nm bandwidth. A 400 nm emission filter was used. Slit dimensions were 0.2 mm width by 6 mm height, so that the slit height completely covered the width of the sample lane. A scanning speed of 0.5 mm/sec was used. Sensitivity was adjusted so that the maximum peak on the plate was near 100% full-scale response of the densitometer.

Peaks were integrated using a SP 4270 integrator (Spectra-Physics, San Jose, CA). Baseline irregularity, incomplete peak resolution and the irregular peak shapes of some complex lipid classes, such as the triacylglycerols and wax esters, often limited the ability of this integrator to accurately detect and quantify compound peaks. More sophisticated peak detection and integration systems (such as the DAS/DYSC system used for GC analyses) should be used to improve the accuracy of baseline detection and peak quantification.

RESULTS AND DISCUSSION

A developed HPTLC plate illustrating the separation of nonpolar lipid classes in simple lipid class standard mixtures and samples of oceanic particles is shown in Figure 1. The major nonpolar lipids are well separated above the preadsorbent zone/silica gel interface. The internal standard cholesteryl formate (CF) is well separated from the sample lipid classes. Monoacylglycerols, glycolipids and polar lipids remain at the interface. Note that while the standard lipid classes that are comprised of a single compound migrate in a tight band, the complex sample

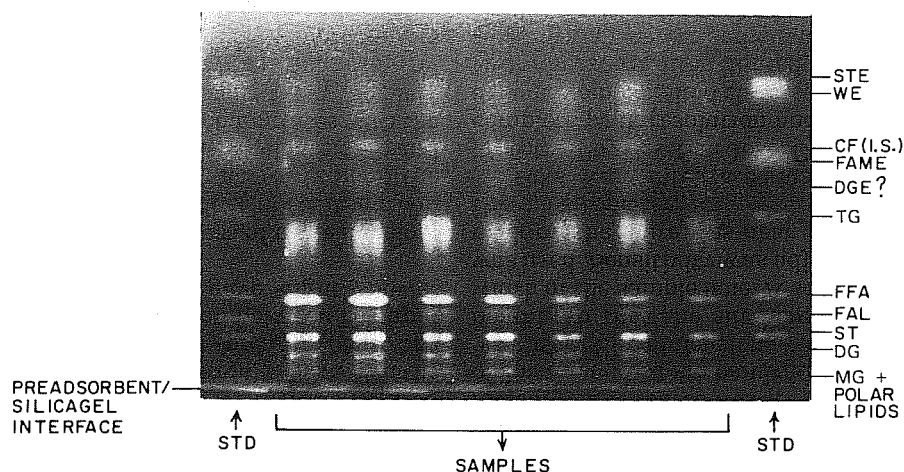


FIG. 1. Developed HPTLC plate showing the separation of nonpolar lipid classes in simple lipid standard mixtures and in samples of oceanic particles from a Slope Water profile taken in June 1982. Development was by Method I, as described in the text. The plate is viewed under UV light and has been overloaded for photographic purposes.

lipid classes migrate in a wide band due to the differences in relative mobilities of the fatty acids composing the lipid class compounds. This effect is especially pronounced for triacylglycerols and wax esters. The fluorescent response is lipid-specific; pigments do not react. In addition, the fluorescence of the different lipid classes show slight variations in color that aid in preliminary identification of sample unknowns.

Although much effort was made to standardize all aspects of the chromatography, variability in compound and background fluorescence often was significant, especially between plates run on separate days. Part of the background fluorescence could be attributed to impurities left in the plate or adsorbed following predevelopment. Lower backgrounds were obtained if the plate was predeveloped just before oven activation and use. Compound response and background fluorescence also is affected by the amount of residual solvent left on the plate. Some residual solvent is necessary for the derivatization reaction (a completely dried plate showed lower compound fluorescence) but too much solvent resulted in a high background. In addition, Schmitz et al. (35) found the development of fluorescence to be strongly time- and temperature-dependent. Therefore, slight differences in heating also may have been an additional source of variation. It is important to note that this interplate variability does not pose a problem in quantification because standards are run on the same plate as samples.

Much of the nonspecific background fluorescence diminished within the first few days, while compound fluorescence remained stable. Better results may be obtained if plates are allowed to sit dessicated for several days before scanning. No loss of sensitivity was detected when plates were rescanned after two months of dessicated storage. Therefore, plates can be stored for extended periods and easily rescanned or reintegrated to optimize quantification without rerunning the sample.

Standard curves. Standard curves demonstrate that nonpolar lipid classes varying by two orders of magnitude may be quantified on the same run. Response vs amount for simple lipid classes were constructed over a range of 5 to 300–700 ng. Two plates with standard lipid mixtures were run.

The first plate covered the range of standard lipid classes from 20–30 to 500–700 ng and was developed by Method I (Fig. 2). Response was proportional to the log of amount throughout the entire range for all classes except for cholesteryl oleate and methyl oleate, which showed linear relationships at concentrations below 100 ng. Standard curves for these compounds thus were split into two segments; a linear fit was used at low levels and a log linear fit at higher levels. Correlation coefficients better than 0.998 for all regression equations except the linear fit of cholesteryl oleate at concentrations <75 ng ($r^2 = 0.982$).

The second plate covered lower concentrations ranging from 5 to 200 ng. This plate was developed by Method II. Background fluorescence was significantly lower than on the first plate. The absolute limit of detection was 5 ng or less for all classes except for triacylglycerol, in which the limit of detection was 10 ng. Plate surface irregularity was the major factor limiting the ability to identify the sample peaks at the lowest levels. Response was a linear function of amount in the range of 5–120 ng

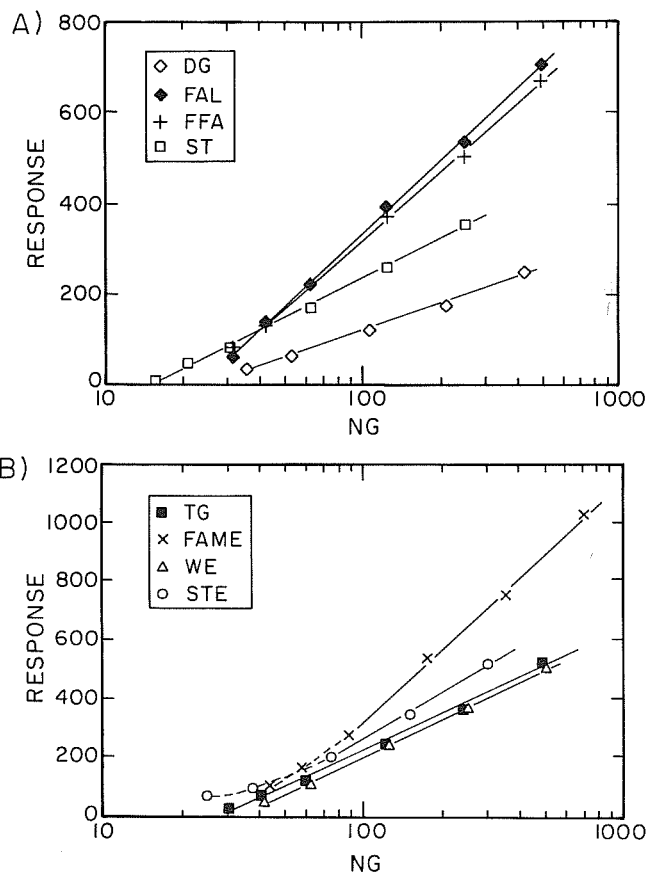


FIG. 2. Standard curves of fluorescent response vs log concentration for simple lipid classes on plate #1. Development was by Method I. Response is in arbitrary units. A) Standard curves for 1,3 diolein, cholesterol, oleyl alcohol and oleic acid; B) standard curves for triolein, methyl oleate, stearyl oleate and cholesteryl oleate.

(Fig. 3). However, response at 200 ng was significantly below that predicted from the linear standard curve, indicating the development of fluorescent quenching. Correlation coefficients of linear regression equations for 5–120 ng were over 0.996 for each standard.

In addition to the shapes of the standard curves, the relative responses of the various lipid classes also differed significantly between the two plates. Cholesterol and oleic acid, in particular, had much lower relative responses on the first plate. Schmitz et al. (35) found that fluorescence of the lipid derivatives increases with time to a maximum and then falls to zero as the derivatives ash. This decrease was found to be more rapid with cholesterol than with triacylglycerol. It is likely that the lower relative response of cholesterol and oleic acid on the first plate may have been due to partial ashing of their derivatives, which suggests that the timecourse of derivatization reactions also is influenced by conditions on the plate itself. As acidic conditions are necessary for fluorescence development, it is possible that slightly higher residual acid on the first plate due to differences in development may have influenced the reaction rates as well as the maximum fluorescence developed. Standardized conditions would reduce the interplate variability observed here. The highly significant standard curves demonstrate that excellent

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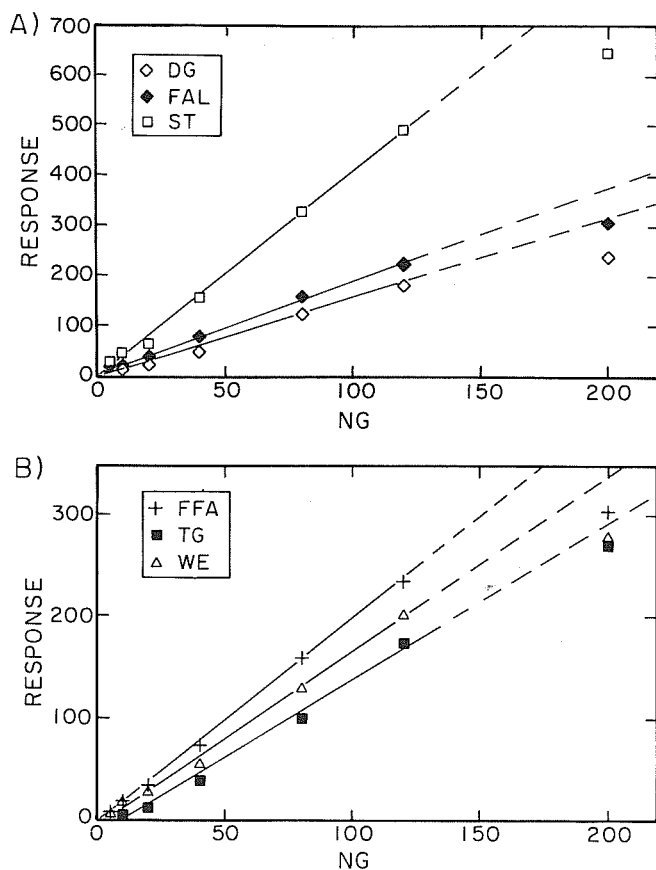


FIG. 3. Standard curves of fluorescent response vs concentration for simple lipid classes on plate #2. Development was by Method II, as described in the text. Response is in arbitrary units. Note the deviation from linearity of standard response at 200 ng, indicating the development of fluorescent quenching. A) Standard curves for 1,3 diolein, cholesterol and oleyl alcohol; B) standard curves for oleic acid, triolein and stearyl oleate.

quantitative results can be obtained throughout a wide range of reaction conditions.

The transition from a linear fit at tens of nanograms concentration to log linear fit at higher concentrations indicates that there is a threshold fluorescent density on the plate above which quenching of the response occurs. This transition from a linear to nonlinear relationship was also observed for lecithin and sphingomyelin derivatives formed by the reaction with HNO_3 vapors (36). Thus, the quenching response may be similar in all fluorescent derivatives, independent of the exact fluorescent inducing reagent.

Differences between the standard curves on the two plates indicate that the exact compound density at which this fluorescence threshold is first observed can vary significantly among plates. The first plate, which had a much higher background, exhibited nonlinearity at a much lower compound density. This suggests that quenching is a function of the overall background fluorescence of the plate as well as the total development of compound fluorescence during the derivatization reaction. These factors may explain the differences in the response vs concentration curves found in this study from that found by Schmitz et al. (35). Thus, external standards must be run

on the same plate and in the same concentration range as sample compounds for accurate quantification. Because the concentration range at which nonlinearity first is observed differs from plate to plate, it is advisable to run several external standards if a wide range of sample concentrations is expected. For this purpose, a 10×20 cm HPTLC plate would allow both a complete standard curve and multiple samples to be developed simultaneously.

Calibration of sample lipid classes with standards. If standard lipid classes, which are comprised of only one compound, are to be used to quantify sample lipid classes, which are comprised of many different compounds, fluorescent response must depend solely on the concentration of lipid class present and not on the exact fatty acid composition of the lipid class. Two experiments were run to test for differences in response between simple lipid class standards and the real complex lipid classes encountered in samples.

The first experiment demonstrated that fluorescence of derivatives is relatively insensitive to both the carbon number and the degree of unsaturation of the fatty acid constituents (Fig. 4). Six fatty acids, ranging from 18 to 22 carbons and from one to six double bonds, were developed on the same plate, and the total responses of 100 ng of each acid derivative determined. No systematic trend of absolute response with either carbon number or number of double bonds was found at this compound density. However, the coefficient of variation among the different acids on the plate was 13.5%, nearly three times that found for the coefficient of variation of sample replicates on different plates. If this variation, in fact, was due to different fatty acid responses, it implies about a 15% accuracy in quantification at this compound density when simple lipid class standards are used. We did not investigate whether this insensitivity to degree of unsaturation varied with compound density on the plate. However, results of this experiment indicate that the MnCl_2 derivatives are less sensitive to lipid class composition than other fluorescent derivatives that show a dependence of fluorescence on component fatty acid unsaturation (34,42,43).

Sample lipid classes also differ from standards in peak geometry on the developed plate. As seen in Figure 1, lipid standards comprised of only one compound migrate in a narrow concentrated zone during development and have well-defined peak shapes. In contrast, complex lipid classes migrate in a wide sample band due to the different relative mobilities of the saturated and highly unsaturated fatty acid constituents of the lipid class compounds. Band broadening alters the compound density of the sample lipid classes on the plate from that of standards; this may significantly affect the amount of fluorescent quenching for a given concentration.

A second experiment demonstrated that these differences in peak shape do not significantly affect the quantification of sample lipid classes (Fig. 5). Fatty acids and triacylglycerols were purified from a large sample of coeanic particulate material. Subsamples from each lipid class were transesterified and then quantified as FAME by capillary gas chromatography to independently determine the concentrations of the sample lipid classes. Three amounts of each sample class, spanning the amounts usually found in samples, were applied to a plate. The

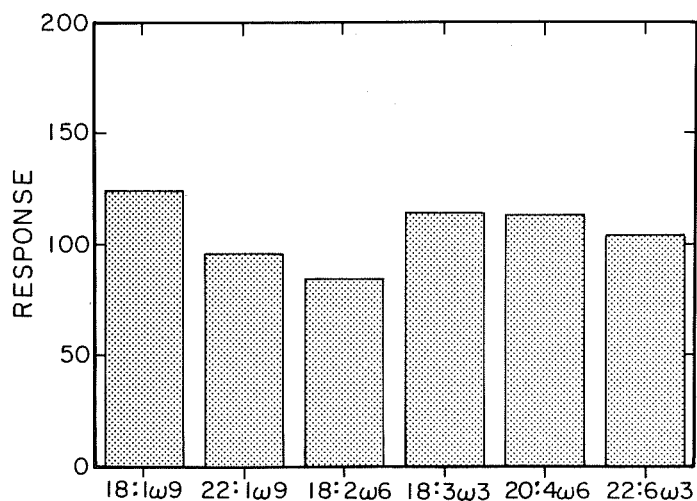


FIG. 4. Relative fluorescent response of 100 ng each of six fatty acids differing in both carbon number and number of double bonds. Development was by Method II. The coefficient of variation was 13.5%. There is no systematic trend of response with either chain length or degree of unsaturation.

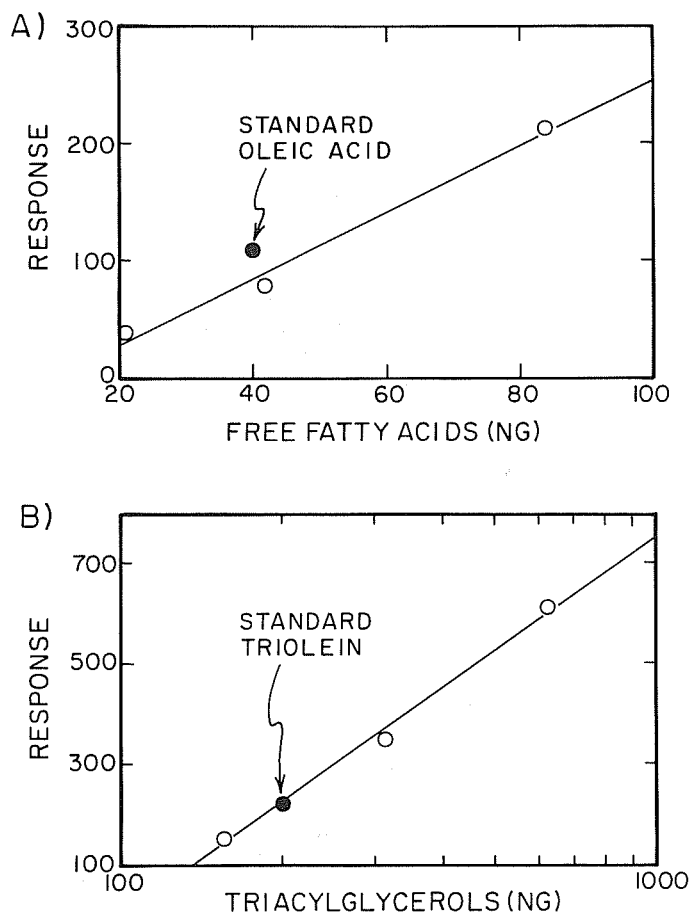


FIG. 5. Calibration of simple lipid standards with complex lipid classes purified from oceanic particulate material. Development was by Method II. The response of simple lipid standards is plotted for comparison against the regression curves calculated for the sample complex lipid classes. A) Fatty acids; B) triacylglycerols.

simple lipid standards oleic acid (FFA) and triolein (TG) were spotted in separate lanes. Regression curves were calculated for the sample lipids using a linear fit for free fatty acids (20–90 ng range) and a log linear fit for TG (150–600 ng range). Estimated amounts of the simple standards oleic acid and triolein calculated using the sample lipid class regression equations were compared with the actual amounts spotted. The standard oleic acid amount was estimated at 46 ng, within 15% of the actual amount of 40 ng spotted. The standard triolein amount was estimated at 198 ng, in excellent agreement with the actual amount of 200 ng. These results demonstrate that although slight differences in fluorescence may exist between simple lipid classes and the complex lipid classes found in samples, standards comprised of a single fatty acid constituent can be used to quantify complex lipid classes if standard and sample concentrations are in the same range. Alternatively, complex lipid mixtures approximating sample lipid class compositions could be used for more accurate quantification.

Application to samples of oceanic particulate material. Oceanic particulate material is an extremely complex environmental sample with plant, animal and microbial sources. There is a wide range of highly unsaturated fatty acids present in the acyl lipid classes. The demands of resolving this complex mixture and the large differences in class concentrations make the determination of lipid class composition in oceanic particles among the most difficult of lipid class analyses.

The HPTLC method was used to resolve and quantify the nonpolar lipid classes in a vertical depth profile of <53 μ m sized oceanic particulate material from the upper 600 meters of the Slope Water in June 1982. A representative chromatogram from a sample at 450 meters is shown in Figure 6. Major lipid classes were sterol, fatty alcohol, free fatty acid, TG, wax ester and a compound tentatively identified as DG. Another minor unidentified lipid, with a R_f similar to that of diacylglycerol ether (DGE) (44), was found just above the TG on the plate. FAME and STE were present only in trace amounts in these samples.

Lipid classes had a wide range of concentration in the same sample. Amounts spotted ranged from a minimum of about 20 ng for free fatty acids to a maximum of 600–700 ng for TG. Replicate sample analyses on separate plates yielded coefficients of variation of 5.9% and 2.3% for sterols (200 ng mean) and TG (300 ng mean), respectively.

The vertical depth profiles of sterol, TG, fatty alcohol and free fatty acid concentration are typical of many bulk property profiles in the upper 1000 meters of the ocean (Fig. 7A,B). These profiles reflect the primary production of particulate organic carbon (POC) in the photosynthetic zone in surface waters and a rapid net loss of POC with depth due to zooplankton grazing and oxidation. However, the wax ester profile (Fig. 7B) does not follow this trend and indicates that a secondary process controls the production and destruction of this compound class in the water column.

A large change is seen in the percent contribution of the individual lipid classes with depth (Fig. 8A,B). Fatty alcohols and free fatty acids both compose less than 5% of the total nonpolar lipids at all depths while sterols, TG, wax esters show large variations. TG (Fig. 8B) decrease

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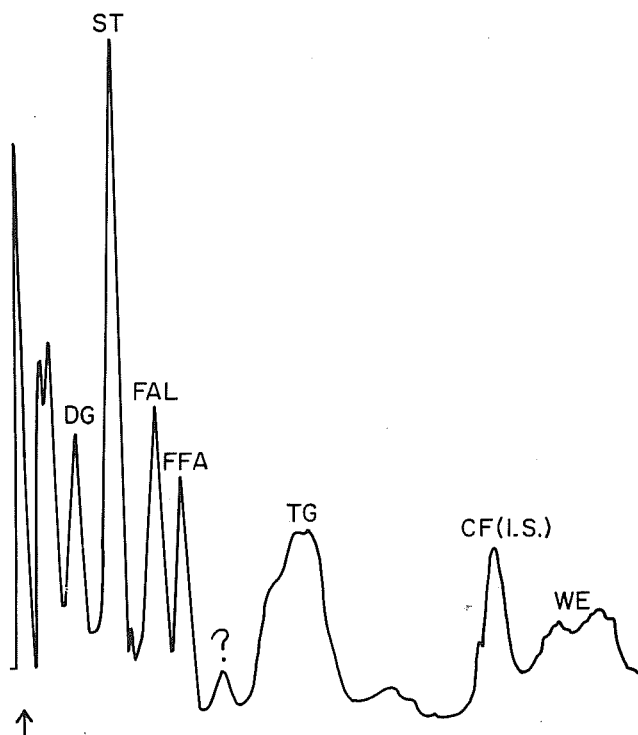


FIG. 6. Chromatogram of nonpolar lipid classes in a sample of oceanic particulate material from 450 m in the Slope Water in June 1982. Development was by Method I. The arrow indicates the preadsorbent zone/silica gel interface.

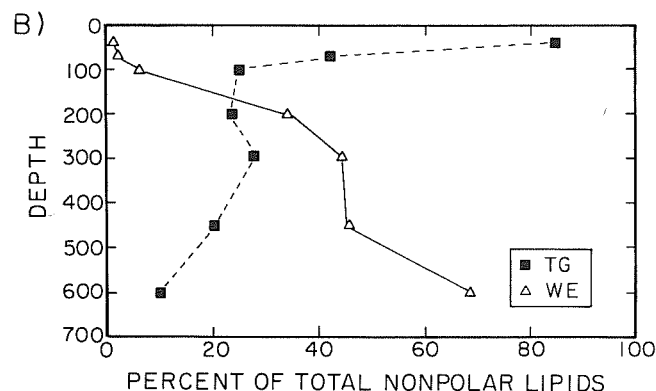
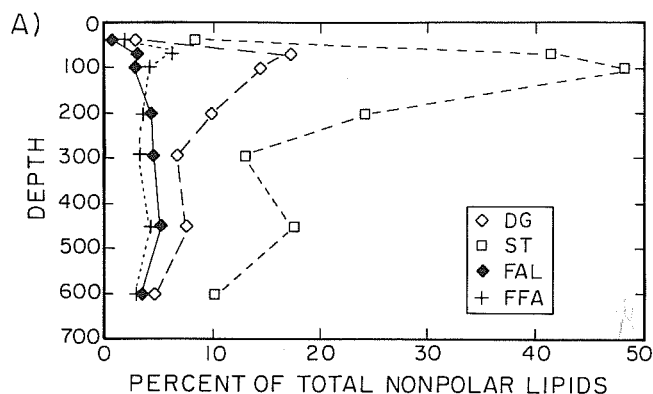


FIG. 8. Relative percentages of nonpolar lipid classes in $<53 \mu$ particulate material in the Slope Water in June 1982.

from over 80% of the total nonpolar lipids at the surface to only 10% in deep water. In contrast, wax esters show the reverse trend and increase from only a few percent of the total nonpolar lipid at the surface to 70% at 600 meters. This change in the dominant particulate lipid class with depth mirrors the general change with depth in the distributions of TG- and wax ester-producing organisms (9,45). Phytoplankton, which dominate the surface waters, synthesize almost exclusively TG. Zooplankton species may synthesize either TG or wax esters, but the major wax ester-synthesizing zooplankton in this temperate region are predominately meso- and bathypelagic. Thus, the increase with depth in the wax ester/TG ratio of the small particle pool most likely reflects changes in relative production of these two compounds by deep-living zooplankton. These results indicate that secondary production of POC by deep water zooplankton significantly alters the suspended particle pool in the water column.

Comparison of HPTLC with other methods. Quantitative HPTLC has several advantages over other methods used in lipid class analyses. The method presented here enables resolution and quantification of all the complex lipid classes in a crude lipid extract when the appropriate standards are run simultaneously on the same plate. Optimal amounts of lipid classes for highest resolution and accuracy in quantification range from 10 to 500 ng, which enables both major and trace lipid class concentrations to be determined from a single run. In addition, the lipid

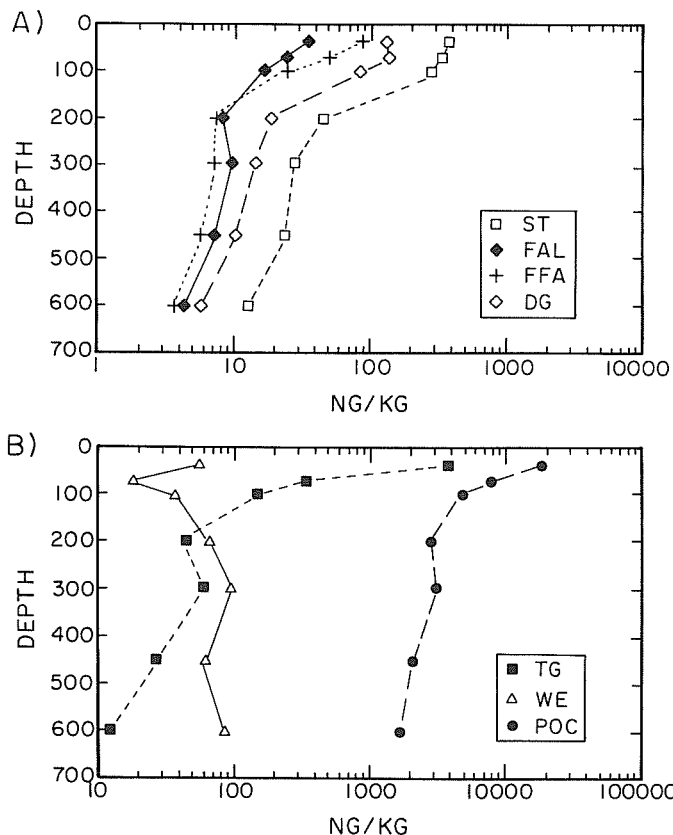


FIG. 7. Nonpolar lipid class concentrations in $<53 \mu$ particulate material in the Slope Water in June 1982. Concentration of total particulate organic carbon (POC) is given for comparison.

specificity of the derivatization reaction in HPTLC minimizes interferences from pigments and other nonlipid compounds present in crude extract. In contrast, the Iatroscan/Chromarod method requires multiple developments and scans for resolution and quantification of all nonpolar lipid classes and requires extensive calibration of the flame ionization detector (FID) response to accurately quantify less than 1 μg of lipid classes.

The chromatographic methods used in this study are very simple and require no specialized equipment. The preadsorbent HPTLC plate eliminates many problems in HPTLC associated with applying very small volumes of crude extracts directly onto the silica gel surface. We have found that a simple, one-step application of dilute crude extracts with a Teflon-tipped pipette and development in standard chambers adequately resolves complex lipid classes. More sophisticated equipment and techniques will increase resolution but also increase cost and effort. Sensitivity in this study primarily was limited not by compound resolution but by the HPTLC plate. Further advances in HPTLC plate technology should continue to improve resolution and reduce the baseline problems associated with the plate surface.

The HPTLC method is rapid and requires no extensive calibration, so over 50 analyses easily can be made in one day. Run-to-run variability is less of a problem with HPTLC than with other methods because standards and samples are run simultaneously. In addition, the static nondestructive detection method allows samples to be scanned many times and in different modes to optimize quantification. In contrast, chromatographic conditions for Iatroscan/Chromarod analyses must be rigidly controlled for accurate results and detection by FID is destructive. Although the scanning densitometer for HPTLC is not inexpensive, it is not dedicated to a single analytical procedure. Therefore, HPTLC may be very cost effective because many diverse HPTLC methodologies may share the same instrument for detection.

We found quantitative HPTLC to be a rapid, simple and very sensitive technique for the analysis of complex lipid class composition in our samples of oceanic particulate material. The method shows much promise for other environmental sample analyses. In addition, the resolution and high sensitivity of the method makes it ideally suited for quantification of lipid classes before preparative scale separations by column chromatography, HPLC or TLC, and for purity checks of lipid fractions.

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